RESEARCH

Dystrobrevin alpha gene is a direct target of the vitamin D receptor in muscle

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Abstract

The biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin D₃ (VD3), exerts its tissue-specific actions through binding to its intracellular vitamin D receptor (VDR) which functions as a heterodimer with retinoid X receptor (RXR) to recognize vitamin D response elements (VDRE) and activate target genes. Upregulation of VDR in murine skeletal muscle cells occurs concomitantly with transcriptional regulation of key myogenic factors upon VD3 administration, reinforcing the notion that VD3 exerts beneficial effects on muscle. Herein we elucidated the regulatory role of VD3/VDR axis on the expression of dystrobrevin alpha (DTNA), a member of dystrophin-associated protein complex (DAPC). In C2C12 cells, *Dtna* and VDR gene and protein expression were upregulated by 1–50 nM of VD3 during all stages of myogenic differentiation. In the dystrophic-derived H2K-mdx52 cells, upregulation of *DTNA* by VD3 occurred upon co-transfection of VDR and RXR expression vectors. Silencing of *MyoD1*, an E-box binding myogenic transcription factor, did not alter the VD3-mediated *Dtna* induction, but *Vdr* silencing abolished this effect. We also demonstrated that VD3 administration enhanced the muscle-specific *Dtna* promoter activity in presence of VDR/RXR only. Through site-directed mutagenesis and chromatin immunoprecipitation assays, we have validated a VDRE site in *Dtna* promoter in myogenic cells. We have thus proved that the positive regulation of *Dtna* by VD3 observed during *in vitro* murine myogenic differentiation is VDR mediated and specific. The current study reveals a novel mechanism of VDR-mediated regulation for *Dtna*, which may be positively explored in treatments aiming to stabilize the DAPC in musculoskeletal diseases.

Key Words

- ▶ vitamin D
- ▶ muscle
- ▶ gene regulation
- ▶ receptor binding

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Introduction

The biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin D₃ (VD3), is a calcium regulating hormone that exerts its tissue-specific biological actions through binding to its intracellular vitamin D receptor (VDR) (Kato 2000). Once VD3 binds to VDR, dimerization with one of the three retinoid X receptors (RXR α , RXR β , and RXR γ) occurs, and the VDR-RXR dimer can translocate to the nucleus where it recognizes specific

genomic sequences called vitamin D response elements (VDREs) to influence gene transcription (Umesono *et al.* 1991, Kliewer *et al.* 1992).

A link of murine VDR implication in transcriptional downregulation of myogenic factors during skeletal muscle development has been initially reported by our team (Endo *et al.* 2003), suggesting that VDR can partially mediate the course of myogenic differentiation.

In vitro studies using murine C2C12 muscle cells backed up the existence of a dose-dependent anti-proliferative effect of VD3 that increased myotube size, which was accompanied by downregulation of myostatin, a negative regulator of muscle mass (Garcia *et al.* 2011, Girgis *et al.* 2014a). The same group demonstrated the presence of an autoregulatory vitamin D-endocrine system in skeletal muscle (Girgis *et al.* 2014a), although the VDR muscle-specific expression was reported to be much lower than that measured in other specific tissues such as intestine (Bouillon *et al.* 2014). VDR expression was found elevated in tibialis anterior muscle obtained from mice treated daily with VD3 via i.m. injection on days 4–7 post-mechanical injury, which suggests the presence of local synthesis and regulation of VD3 metabolism during muscle regeneration (Srikuea & Hirunsai 2016). Furthermore, *Vdr* ablation resulted in reduced grip strength and dysregulation of myogenic factors in mice mimicking atrophic phenotype changes mainly attributed to reduced expression of genes associated with calcium handling channels (Girgis *et al.* 2015). However, in the global VDRKO model, it is hard to discriminate effects of direct VDR deletion in muscle and those caused by mineral metabolism changes such as hypocalcaemia. Modifications in intracellular calcium levels by VD3 can be unavoidable even in conditional VDR null mice such as the myocyte-specific VDR knockout mice described recently (Girgis *et al.* 2019) and interfere with muscle relaxation (Berchtold *et al.* 2000) or tamper with myoblast nature due to the disrupted regulation of phosphate metabolism (Bellido & Boland 1987). It is thus highly probable that the so-called VDR-muscle generated effects observed previously are mediated by tissues other than muscle or even by non-genomic mechanisms related to the disrupted calcium uptake and thus are not a direct effect of muscle *Vdr* ablation *per se*.

Dystrobrevin alpha (DTNA) is an essential structural component of the dystrophin associated protein complex (DAPC). The DAPC maintains sarcolemmal stability during muscle contraction and through its binding domains and associated channel proteins aids signal transduction toward muscle fibers to mainly protect them against damage from stress (Blake *et al.* 2002, Ehmsen *et al.* 2002). The core member of DAPC, dystrophin, serves as an anchor between the actin cytoskeleton and the connective tissue (Ervasti & Campbell 1991, Petrof *et al.* 1993, Ohlendieck 1996). Mutation on dystrophin gene leads to the X chromosome-linked, progressive, fatal degenerative muscle disorder called Duchenne muscular dystrophy (Hoffman *et al.* 1987). The C terminus of DTNA is strongly associated with the C terminus of dystrophin through a specific coiled-coil

motif interaction (Sadoulet-Puccio *et al.* 1997), whereas its N-terminal domain directly interacts with sarcoglycan and sarcospan subcomplex (Yoshida *et al.* 2000). Absence of C terminal dystrophin binding motif does not prevent DTNA binding to the DAPC, indicating that an unidentified anchoring site is present in the N-terminal region of Dtna (Crawford *et al.* 2000). In addition, DTNA colocalizes at the sarcolemma with dysbindin (Benson *et al.* 2001), at the neuromuscular junction with syncoilin (Newey *et al.* 2001), and binds desmuslin (Mizuno *et al.* 2001) and syntrophins (Ahn *et al.* 1996).

The murine *Dtna* gene is located on chromosome 18 consists of 24 coding exons and is subjected to extensive alternative splicing that produces tissue-specific isoforms (Blake *et al.* 1996, Ambrose *et al.* 1997). Overall, three main isoforms have been described in mouse (*Dtna1*, *Dtna2*, and *Dtna3*); however, slight rearrangements within the internal exon usage in individual isoforms generate multiple transcript variants encoding for the same isoform, further adding to the complexity of *Dtna* genomic region (Böhm *et al.* 2009). The largest isoform, DTNA1, is an 87 kDa protein (77–84 kDa in mouse) originally discovered in postsynaptic membranes of *Torpedo* electric organ (Wagner *et al.* 1993), is enriched at the neuromuscular junction, and possesses two syntrophin binding domains, a dystrophin/utrophin binding site as well as a unique COOH-terminal containing three potential tyrosine phosphorylation sites (Peters *et al.* 1998, Pawlikowski & Maimone 2009, Gingras *et al.* 2016). DTNA2 exists in two alternative isoforms (DTNA2A and DTNA2B), possesses a unique C terminal domain, localizes in the sarcolemma and the neuromuscular synapse, and binds only dystrophin (Enigk & Maimone 1999). The smallest DTNA3 isoform (42 kDa) contains two structural motifs belonging to the dystrophin-family proteins, lacks the syntrophin binding domain (Ponting *et al.* 1996), and it is exclusively found in cardiac and skeletal muscle (Nawrotzki *et al.* 1998). The exact function of DTNA is unknown, but it has been speculated that, apart from its supportive role, it may aid in the transmission of DAPC signaling (Newey *et al.* 2000).

The identification of VDR in muscle tissue hints at the presence of a direct pathway for VD3 to impact on skeletal muscle function that is yet to be discovered. Herein, we have shown that VD3 administration positively regulates *Dtna* gene and protein expression in C2C12 myogenic cells as well as in H2K-mdx52 that are immortalized cells derived from the humanized *mdx52* mouse model (Araki *et al.* 1997). DTNA upregulation was strictly associated with VDR expression levels in both healthy and

dystrophic myotubes. We have also identified a functional VDRE in Dtna muscle promoter. This study demonstrates direct regulation of a muscle-specific target through VD3 administration and may have important implications in combination treatment regimes in dystrophinopathies.

Materials and methods

Cell culture

C2C12 mouse myoblast cell line was purchased from ATCC (CRL®1772) and maintained in growth medium (GM) consisting of DMEM-F12 (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco) at 37°C under 5% CO₂. Upon reaching 70% confluence, cells were seeded in appropriate plates and cultured in GM in the presence of vehicle (ethanol) or VD3 (FC09794, Carbosynth, Japan) (myoblast assay). To induce *in vitro* myogenic differentiation, when seeded cells were 80% confluent, GM was replaced with differentiation medium (DM) consisting of DMEM-F12 supplemented with 1.5% horse serum (HS), (Gibco) according to established protocols (Fujita *et al.* 2010). The DM containing the appropriate amount of vehicle or VD3 was replenished daily.

Mouse H2K-mdx52 myoblasts were generated by crossing H-2Kb-tsA58 female with *mdx52/mdx52* F1 male mice to yield dystrophin-deficient H2K-mdx52 myoblasts (Jat *et al.* 1991, Morgan *et al.* 1994). H2k-mdx52 myoblasts were grown on gelatin-coated dishes in DMEM GlutaMAX (Gibco) GM supplemented with 20% (v/v) FBS, 20 U/mL murine interferon- γ (Peprotech), 2% chick embryo extract (US Biological), 2% L-Glutamine (Gibco), and 1% penicillin-streptomycin at 33°C under 5% CO₂. Upon reaching 80% confluence, cells were differentiated by switching to DMEM with GlutaMAX containing 2.5% (v/v) HS (day 0) at 37°C under 5% CO₂.

Neuro-2a cells were purchased from ATCC (CCL-131). Cells were maintained in GM consisting of EMEM (ATCC 30-2003) supplemented with 10% (v/v) FBS. Cells were divided into appropriate plates for assay and cultured in GM with or without VD3.

For all cells, images were acquired with Olympus U-RFL-T, TH4-100 microscope using Aquacosmos NAF camera software.

Real-time quantitative PCR analysis (RT-qPCR)

Total RNA was extracted from cultured cells using QIAGEN RNeasy mini kit. 500 ng of RNA was used as

template for RT using ReverTra Ace® qPCR RT Master Mix with gDNA Remover kit (Toyobo, Japan) according to the manufacturer's instruction. The RT cDNA reaction products were subjected to quantitative real-time PCR using SYBR green PCR master mix kit (Applied Biosystems) and a StepOne Plus Real-time PCR (Thermo Fisher Scientific). The protocol included melting for 10 min at 95°C and 40 cycles of 2-step PCR (melting for 15 s at 95°C and annealing/elongation for 1 min at 60°C). Optimal primer sequences were designed using Primer-BLAST (Supplementary Table 1A, see section on [supplementary materials](#) given at the end of this article). The relative fold change was calculated by using the formula $2^{-\Delta\Delta C_t}$. In all experiments, glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and cyclophilin A were used as housekeeping genes.

siRNA knockdown

C2C12 cells at an appropriate density (60% confluency) at 24-well plate were supplemented with 10 nM siRNA for each target using Lipofectamine RNAiMAX (Thermo Fisher Scientific) in GM for 24 h. Subsequently, the GM was changed to DM with vehicle or VD3 and cells were incubated for 48 h. The knockdown efficiency was ascertained by RT-qPCR and/or Western blotting. To exclude off-target effects, two different duplexes were used for each target as follows: MyoD1 (SR-410431, Origene), Dtna1 (sc-43323, Santa Cruz) and (SR-419093, Origene), VDR (sc-36811, Santa Cruz), and VDR (AM16708, Thermo Fisher Scientific).

Generation of plasmids

Full-length cDNA of 5'-terminally pFLAG-tagged mouse VDR (GenBank: D31969) was cloned into the pcDNA3 (Invitrogen) vector between EcoRI and XbaI sites. Mouse RXR alpha expression vector pSG5-RXR α was previously described (PMID: 16380173). Dtna promoter fragments were retrieved from genomic DNA extracted from C57BL/6 mice and cloned into pGL4 vector (Promega). Primer-specific PCR fragment was generated using KOD plus polymerase (Toyobo) and an Applied Biosystems 96-well PCR Thermocycler. Primers were generated by Primer-Blast and purchased by Eurofins (Supplementary Table 1B). Verification of insert was executed by sequencing (Eurofins).

Transient transfections

Transient transfections of VDR/RXR plasmids in Neuro2a cells were performed with Lipofectamine 3000

(Invitrogen) according to the manufacturer's instruction. 150 ng of pFLAG-mVDR, 150 ng of pSG5-mRXR α , or their equivalent empty vector (mock transfection) were transfected in 60% confluent cells seeded in 24-well plates in EMEM medium without antibiotics.

Plasmid transfection in H2K-mdx52 myoblasts was performed via electroporation using Amaxa Cell Line Nucleofector Kit L and program D-023 according to the manufacturer's instruction. 0.6 μ g of pFLAG-mVDR, 0.6 μ g of pSG5-mRXR α , or empty vector were transfected in H2K-mdx52 cells per well (12-well) plate.

Site-directed mutagenesis

Screening and identification of VDRE in Dtna cloned vectors were executed *in silico* with LASAGNA-Search 2.0 (Lee & Huang 2013) and JASPAR_CORE 2018 database (Khan *et al.* 2018). In order to generate point mutations in the putative VDREs in the promoter region of Dtna gene, 5 ng of original plasmid were used in a KOD plus standard PCR reaction containing the set of mutagenesis primers (Supplementary Table 2A). The PCR product was digested with DpnI for 1 h at 37°C (10 U/ μ L). Kination reaction was as follows: 20 μ L of gel-extracted PCR produce, 2 μ L of 10 X T4 polynucleotide kinase buffer (NEB), 0.5 μ L of T4 PNK kinase enzyme (NEB), and 0.5 μ L of rATP 100 mM (Promega) at 37°C for 1 h. 10 μ L of the product were ligated with 10 μ L mighty mix ligase (Takara) for 2 h at 16°C and the transformation in LB/Ampicillin plates followed. Mutagenesis was verified by sequencing (Eurofins).

Dual luciferase assay

C2C12 cells were transfected when 70–80% confluent with 500 ng Dtna-Luc plasmid or control pGL4 (promoterless vector) and 35 ng of pTK-RLuc renilla plasmid (Promega) using Lipofectamine 3000 (Invitrogen). Twenty-four hours later, GM was replaced with DM in presence of VD3 or ethanol.

Neuro2a cells were transfected when 70% confluent with 500 ng Dtna-Luc plasmid or control pGL4 and 10 ng of renilla plasmid using Lipofectamine 3000. For VDR/RXR co-transfection, 150 ng of pFLAG-mVDR, 150 ng of pSG5-mRXR α , 200 ng of Dtna-Luc, and 10 ng of pTK-RLuc were used. Twenty-four hours later GM was supplemented with VD3 or ethanol.

Cells lysates were collected according to manufacturing instructions of Dual-Luciferase Reporter

Assay Kit (Promega). The luciferase activity was detected using a GloMax Navigator System GM2010 (Promega).

Western-blot analysis

Cells were collected in RIPA buffer (Thermo Fisher Scientific) containing protease inhibitors (Roche) and sonicated with a Branson 250D sonicator. Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of protein (15–20 μ g) were mixed with NuPAGE LDS Sample Buffer containing 2% NuPAGE sample reducing agent (Thermo Fisher Scientific), denatured at 70°C for 10 min, subjected to SDS-PAGE using a Biorad 4–20% precast gel at 150 V for 55 min in Tris-glycine buffer or NuPAGE 3–8% precast gel at 150V for 75 min in Tris-acetate buffer, and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon) using a semi-dry blotting system for 30 min at 0.19 A in AE-1460 EzBlot buffer or EZ Fast Blot HMW buffer (Atto Co). The PVDF membranes were blocked in 5% skimmed milk and immunoblotted with the following primary antibodies overnight at 4°C: anti-DTNA (rabbit recombinant monoclonal, EPR14112, 1/500 from Abcam) or anti-DTNA1 (rabbit monoclonal, 1/500, in-house-made, (Yoshida *et al.* 2000), anti-VDR (mouse monoclonal, D-6, 1/500 from Santa-Cruz) or anti-VDR (rabbit recombinant monoclonal, EPR4552, 1/500 from Abcam), anti-myosin skeletal slow (mouse monoclonal, M-8421, 1/200 from Sigma) or anti-myosin heavy chain type I (mouse monoclonal, BA-D5, 1/200 from DSHB), anti-myosin skeletal fast (mouse monoclonal, M-4276, 1/500 from Sigma), and GAPDH (mouse monoclonal, MAB374, 1/500 from Chemicon). Histofine Simple Stain MAX-PO (1/200, Nichirei Bioscience Inc., Japan; 424151) was used as secondary antibody. Proteins were visualized by the ECL Prime Western Blotting Detection Reagent (GE Healthcare; RPN2232) and a ChemiDoc MP Imaging System (Bio-Rad) and were analysed using Image Lab 6.0 (Bio-Rad).

Chromatin immunoprecipitation (ChIP) assay

Chromatin was extracted using Abcam chromatin extraction kit (ab117152). The lysate was sonicated to shear DNA into fragments of 200–1000 bp (12 cycles of 20 s sonication, 40 s pausing). ChIP sample preparation was performed by the High Sensitivity ChIP kit (Abcam, ab185913) and VDR antibody (D-6, Santa Cruz). One millilitre of the immunoprecipitated sample was subjected to PCR analysis using ExTaq polymerase (Takara) and

primers flanking mouse *Dtna* and *Gapdh* promoter regions (Supplementary Table 2B).

Statistical analysis

All data are expressed as means $\pm$ S.E.M. Statistical analysis was performed using Graph Pad Prism version 8.04 for Windows. Comparison between two groups was assessed by unpaired *t*-tests and more than two groups were assessed by the one-way ANOVA with Tukey or Sidak's post-hoc test or two-way ANOVA with Sidak's post-hoc analysis. Probabilities less than 5% ($*P < 0.05$), 1% ($**P < 0.01$), 0.1% ($***P < 0.001$), or ($****P < 0.0001$) were considered to be statistically significant.

Results

VD3 treatment upregulates mRNA levels of *Dtna* during all stages of C2C12 differentiation

In order to investigate the molecular mechanism of *Dtna* regulation by the VD3/VDR axis, we used the mouse C2C12 myogenic cell line, a well-established *in vitro* model for studying myogenic differentiation (Burattini *et al.* 2004). Moreover, C2C12 cells express relatively high levels of VDR, an essential prerequisite to investigate potential VD3/VDR axis mediated specific gene effects (Girgis *et al.* 2014b). We were able to distinguish early myotubes on day 3 of differentiation, whereas on day 5 almost all cells had undergone cell fusion to form mature myotubes (Supplementary Fig. 1). It has been reported that administration of VD3 during C2C12 differentiation upregulates mRNA levels of *Vdr* and *Cyp24a1* (the key enzyme responsible for the catabolism of VD3) and downregulates myogenin and myostatin (Girgis *et al.* 2014a), effects that we have confirmed in our system (data not shown). Because supraphysiological doses or prolonged administration of VD3 are prone to exert anti-proliferative effects in C2C12 cells or mask VD3-direct effects on genes due to modulation of non-genomic pathways, we chose to supplement cells with low doses of VD3 throughout the three distinct phases of differentiation rather than spanning all differentiation period: myoblasts, early myotubes (day 0–3), or mature-formed myotubes (day 3–5). Administration of 10 nM of VD3 in myoblasts undergoing differentiation (day 0–2) upregulated almost all *Dtna* transcript variants detected by qRT-PCR (Supplementary Fig. 2). For gene pattern analysis, we chose to focus on *Dtna* due to its ability to bind both dystrophin and utrophin, proteins that are differentially

regulated in dystrophic mice. Furthermore, we were able to clone the transcript 2 version of *Dtna1* from our C2C12 cells and overexpress it in order to back up our data (results not shown). All subsequent qPCR studies described in this paper were carried out using primers specific for *Dtna1*, although primers designed for the detection of *Dtna1*, *Dtna2*, and the canonical (longest) isoforms produced identical results. We first demonstrated that mRNA levels of endogenous *Dtna1* were significantly upregulated upon administration of 10 nM in myoblasts (Fig. 1A) and early myotubes (Fig. 1B) at all intervals examined (12 h, 24 h, and 48 h). In myotubes, *Dtna1* levels were markedly raised throughout the 12 h and 24 h examined period and upon addition of 100 nM rather than 10 nM of VD3, possibly due to the fact that VDR expression steadily declines throughout the course of myogenic differentiation, and thus higher concentrations of VD3 are required in order to exert an effect on target genes (Fig. 1C). To ascertain that the observed *Dtna1* upregulation was VD3-mediated, we next performed a dose-response assay using the 48 h interval that gave us significant increases at all periods examined. In myoblasts, administration of 5 and 10 nM VD3 yielded a significant increase of *Dtna1* (Fig. 1D), and in early myotubes *Dtna1* response to VD3 was significant at all concentrations examined (Fig. 1E), and finally in late myotubes, as already deduced from the time-course data, 10 and 50 nM of VD3 were required in order to produce a significant effect (Fig. 1F). Collectively the previously mentioned data indicate that *Dtna1* elevation during *in vitro* C2C12 differentiation is VD3-dependent.

VD3 treatment upregulates protein levels of DNTA and VDR during all stages of C2C12 differentiation

As a next step, we wished to confirm whether this *Dtna* VD3-mediated regulation could lead to an increase in DTNA protein levels concomitantly with an increase in VDR protein levels. In myoblasts treated with 10 nM VD3 for 48 h, DTNA and VDR protein levels were significantly upregulated (Fig. 2A), whereas in early myotubes, 1 nM of VD3 administration for the same time frame produced an identical effect (Fig. 2B). In myotubes, treatment with 10 nM VD3 for 48 h upregulated DTNA, VDR as well myosin skeletal fast (MSF), and the slow type myosin isoform (myosin skeletal slow, MSLOW), confirming the *in vitro* anabolic effects of VD3 on differentiation previously described (Okuno *et al.* 2012). A dose-response Western blotting verification utilizing VD3 concentrations ranging from 5 nM to 50 nM was conducted for myotubes (Fig. 2D) and the quantitative data obtained backed up the qPCR

Figure 1

VD3 dose-dependently increases *Dtna1* transcript levels during different stages of C2C12 myogenic differentiation. Left panel: Time course of *Dtna1* induction upon VD3 administration. mRNA levels of endogenous *Dtna1* in (A) myoblasts upon administration of 10 nM VD3 for 12–48 h in GM; (B) early myotubes upon administration of 10 nM VD3 for 12 h (day 1, overnight), 24 h (day 1–2), and 48 h (day 0–2) in DM; and (C) myotubes upon administration of 10 or 100 nM VD3 for 12 h (day 4, overnight), 24 h (day 4–5), and 48 h (day 3–5) in DM. Right panel: Dose-response of VD3-induced *Dtna1* expression in C2C12 myoblasts, early myotubes, and myotubes. C2C12 cells were stimulated with 1–50 nM VD3 for 48 h in the presence of (D) GM (myoblasts), (E) DM at day 0–2 (early myotube), or (F) DM at day 3–5 (myotube). Day 0 represents the start date of differentiation. Data are expressed as mean $\pm$ s.e.m. Expression of mRNA was quantified by qRT-PCR and normalized using cyclophilin A and *Gapdh*. Comparison between the treated and untreated group was performed using unpaired two-tailed *T*-test. Comparison between different VD3 treatment regime vs vehicle was performed using ordinary one-way ANOVA with Sidak's post-hoc test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$. $n = 3$ individual experiments performed in triplicate.

data (Fig. 1F). Collectively the previously mentioned data indicate that low doses of VD3 upregulate DTNA and VDR at all stages of myogenic differentiation.

DTNA upregulation in C2C12 myotubes is VDR mediated and specific

We then wished to examine whether the observed *Dtna* upregulation in myogenic cells was indeed mediated by VDR or could be related to VD3 non-genomic or

Figure 2

DTNA and VDR protein levels are upregulated in VD3 treated C2C12 cells. (A) Western blot analysis and densitometric quantification of DTNA and VDR protein levels in C2C12 myoblasts treated with 10 nM VD3 for 48 h in the presence of GM. (B) Western blot analysis and densitometric quantification of DTNA and VDR protein levels in early myotubes treated with 1 nM VD3 for 48 h (day 0–2) in presence of DM. (C) Western blot analysis and densitometric quantification of DTNA, VDR, Myosin skeletal fast (MSF) and Myosin skeletal slow (MSLOW), protein levels in C2C12 myotubes treated with 10 nM VD3 for 48 h (day 3–5). (D) Western blot analysis and densitometric quantification of DTNA protein levels in C2C12 myotubes treated with 5, 10, or 50 nM VD3 for 48 h (day 3–5). Data are representative of $n = 3$ individual experiments. Quantification data are expressed as mean $\pm$ s.e.m. Comparison between treated and untreated group was performed using unpaired two-tailed *T*-test. DTNA dose-response data were compared using ordinary one-way ANOVA with Sidak's post-hoc test against vehicle. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$.

non-nuclear receptor mediated effects (Hii & Ferrante 2016, Hirota *et al.* 2017). As the most prominent *Dtna* and *Vdr* changes upon VD3 administration occurred during early stages of *in vitro* differentiation, we performed the following siRNA assays in early myotubes treated with 10 nM VD3 for 48 h. We first confirmed the enhanced *Vdr* expression by VD3 and reduction of *Dtna1* induction by VD3 upon siRNA *Vdr* knockdown (Fig. 3A). Subsequently, we silenced *MyoD1*, a transcription factor that is

well-known to bind to E-box motifs and to mediate regulation of muscle-specific genes (Shklover *et al.* 2007), and confirmed that such interference did not abolish the *Dtna1* induction by VD3 in our system (Fig. 3B). We finally silenced *Dtna1* itself, an event which was consistent with a decreased *Dtna1* mRNA expression. The *Dtna1* knockdown was independent on the *Cyp24a1* induction (Fig. 3C) or VDR protein upregulation by VD3 (Fig. 3D), excluding any off-target effects of the silencing duplex

and confirming that the observed DTNA induction has probably occurred downstream of VDR:RXR binding. Collectively the previously mentioned data indicate that the *Dtna* gene and protein upregulation in C2C12 cells is VDR mediated and specific

The induction of DTNA by VD3 in dystrophic-derived H2K-mdx52 cells is VDR mediated and specific

We then wished to further check whether the DTNA response to VD3 is VDR mediated by using the H2K-mdx52 murine cells that lack dystrophin. In the H2K-mdx52 cell line, VDR levels are barely detectable by Western blotting, and after conducting several dose-response assays we chose to treat cells with 100 nM VD3 daily because only such a concentration was capable of substantially increasing the endogenous VDR levels at a short-term (48 h, Fig. 4A) or longer-term administration (5 days, Fig. 4B) consistently at all our assays. VDR levels were elevated in early myotubes mock-transfected with empty pFLAG vector and treated with VD3 for 48 h; however, this elevation was not enough in order to aid DTNA protein elevation as well. Harvesting the cells 72 h rather than 48 h after the final addition, mildly but not significantly, elevated DTNA protein levels (Supplementary Fig. 3A). Equally, addition of 100 nM of VD3 throughout the whole differentiation period (day 0–5) has elevated *Dtna1* mRNA levels (data not shown), but failed to significantly elevate protein levels (Fig. 4B). We also performed a VD3 dose-response assay, but even at a 100 nM concentration that raised VDR approximately 7 fold (the highest fold raise we have observed), we failed to see DTNA upregulation, indicating that endogenous VDR levels are too low to achieve this effect (Supplementary Fig. 3B). When H2K-mdx52 myoblasts were co-transfected with mVDR/RXR plasmids, significantly increased DTNA and VDR levels were detected upon treatment with 10 nM VD3. Furthermore, a 17-fold elevation in VDR levels in mVDR/RXR co-transfected cells vs the mock-transfected ones has significantly raised DTNA levels upon treatment with 100 nM VD3 for the same time frame (Fig. 4A). Interestingly, VD3 administration led to the upregulation of MSLOW levels rather than MSF levels in H2K-mdx52 generated myotubes (Fig. 4B), indicating that slow fiber marker upregulation by VD3 does not depend on VDR protein levels and may be due to an indirect-VD3 mediated effect or to a non-genomic response. Finally, a dose-response assay performed with H2K-mdx52 cells co-transfected with mVDR/RXR plasmids and treated with VD3 every alternative day throughout the differentiation

Figure 3

The *Dtna* response to VD3 in C2C12 early myotubes is VDR mediated and specific. (A) *Vdr* ablation by siRNA successfully downregulates *Vdr* and concomitantly suppresses *Dtna1* induction upon VD3 administration. On the contrary, (B) *MyoD1* ablation via siRNA has no effect on the induction of *Dtna1* by VD3, and (C) *Dtna1* siRNA mediated knockdown leads to loss of *Dtna* gene induction by VD3 without alteration in *Cyp24a1* expression. (D) Western blot performed in *Dtna1*-ablated differentiating myoblasts demonstrates reduced DTNA protein expression without alteration in VDR endogenous expression as clearly shown by protein quantification. C2C12 cells were transfected with the respective siRNAs or scramble controls using Lipofectamine RNAiMax (Invitrogen) in the presence of GM and after 24 h were treated with VD3 (10 nM) for 48 h in the presence of DM. Expression of mRNA was quantified by qRT-PCR and normalized using *Gapdh*. Data are expressed as mean $\pm$ S.E.M. and were compared using ordinary one-way ANOVA with Sidak's multiple comparison test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$, NS: not significant. $n = 3$ individual experiments with two different siRNA sequences used to confirm the *Dtna1*, *MyoD1*, and *Vdr* silencing effect.

Figure 4

DTNA response to VD3 is dependent on VDR expression levels in H2K-mdx52 myogenic cells. (A) VDR but not DTNA protein levels were upregulated in H2K-mdx52 early myotubes that were mock-transfected with pFLAG and treated with 100 nM VD3 for 48 h in the presence of DM. However, both DTNA and VDR were upregulated by 100 and 10 nM VD3 for 48 h in presence of DM upon co-transfection of mVDR/RXR plasmids via electroporation as described in materials and methods. Comparison between four groups was done by one-way ANOVA with Tukey post-hoc test. Comparison between 10 nM treated and untreated group was performed using unpaired two tailed *t*-test. (B) DTNA and MSF protein levels were unaltered in H2K-mdx52 myotubes treated with 100 nM VD3 daily for 5 days during myogenic differentiation, whereas MSLOW was slightly elevated as clearly demonstrated in respective quantification. VDR level was elevated in VD3 treated cells. Comparison between treated and untreated group was performed using unpaired two tailed *t*-test. (C) DTNA was dose-dependently upregulated in H2K-mdx52 myotubes, co-transfected with mVDR/RXR plasmids, and treated with either 5, 10, or 50 nM VD3 every alternative day throughout the differentiation period. Quantification data are expressed as mean $\pm$ S.E.M. Comparison between groups was performed using ordinary one-way ANOVA with Sidak's multiple comparison test. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$. NS: not significant.

period yielded results similar to C2C12 (Figs 4C vs 2B). These data further strengthen the hypothesis that Dtna gene is a direct target of the VD3/VDR axis rather than being activated by intermediate VD3 induced pathways or transcription factors. Furthermore, since a modest but significant VDR upregulation failed to raise DTNA protein levels in H2K-mdx52 myotubes, we presume that

a certain threshold of VDR expression is required in order to achieve DTNA upregulation.

The muscle-specific Dtna promoter is activated by VD3

In order to ascertain direct regulation of *Dtna* through VD3/VDR axis, we wished to generate a muscle-specific *Dtna* promoter and assess whether its activity could be enhanced in the presence of VD3. We have based our *Dtna* promoter design in Holzfeind *et al.* and named the three promoter regions that we cloned according to the original paper: *Dtna*-M (muscle), *Dtna*-B (Brain), and *Dtna*-LB (Lung/Brain). For all three promoters, we have included the transcription start site (TSS) described in original publication; however, we have further extended their 5'-upstream sequence in an attempt to include putative VDRE regions that we have identified via the LASAGNA and JASPAR tool (Fig. 5A). We first checked the specificity of individually cloned promoters in our C2C12 system. In early myotubes supplied with 10 nM VD3 for 48 h, *Dtna*-M promoter yielded the highest basal activity, followed by *Dtna*-LB, whereas *Dtna*-B was somewhat active (Fig. 5B). This observation was not surprising, taking into account that *Dtna*-M promoter possesses three E-box motifs (CANNTG) and a myogenin binding site, whereas the other two promoters do not. Evaluation of the dose-response of *Dtna*-M promoter upregulation upon 48 h of 1 nM-10 nM of VD3 supplementation was successful (Fig. 5C). To double confirm the eligibility of *Dtna*-M as muscle-specific promoter in our system and its VDR-mediated upregulation, Neuro2a cells, that have very low levels of VDR, were used to assess basal activity of all three promoters. In these cells, we found that *Dtna*-B promoter had the highest basal activity, followed by *Dtna*-M promoter, whereas *Dtna*-LB promoter was inert (Supplementary Fig. 4A), and as expected, addition of VD3 had no effect on the induction of neither *Dtna*-M or *Dtna*-B. On the contrary, in the presence of overexpressed VDR/RXR, *Dtna*-M induction by VD3 was deemed to be significant, but no such induction was observed for *Dtna*-B (Supplementary Fig. 4B). Successful VDR transfection in these cells was confirmed via Western blotting (Supplementary Fig. 4C). The promoter assay was repeated in H2K-mdx52 cells, where only *Dtna*-M exhibited elevated basal activity but no induction in presence of VD3 was observed (data not shown). Collectively the previously mentioned data indicate that the *Dtna*-M is indeed a muscle-specific promoter and that its activation by VD3 is VDR mediated.

Figure 5

Generation of Dtna-muscle specific promoter. (A) Schematic representation of intron-exon variants of *Dtna* gene located in chromosome 18 and putative promoter regions of Dtna-M (muscle), Dtna-B (brain), and Dtna-LB (lung-brain) cloned in a pGL4 promoterless luciferase vector cassette. Exons are depicted as blue squares. TSS: transcription start site; ORF: open reading frame. (B) Dual luciferase assay indicates high Dtna-M basal promoter activity that is significantly upregulated in the presence of 10 nM VD3 in C2C12 early myotubes. Comparison between groups was done by 2-way ANOVA with Sidak's multiple comparison test. Data are expressed as mean $\pm$ S.E.M. and are representative of three individual experiments performed in triplicate. (C) Dtna-M promoter is dose-dependently upregulated upon administration of 1–10 nM VD3. Data are expressed as mean $\pm$ S.E.M. and were compared using ordinary one-way ANOVA with Sidak's post-hoc test. $n = 3$, $*P \leq 0.05$, $***P \leq 0.001$, and $****P \leq 0.0001$. Constructs were transfected in C2C12 cells that were incubated in DM in the presence of vehicle or VD3 (10 nM) for 48 h and the measured firefly luciferase activity was normalized to renilla luciferase. Promoterless pGL4 activity was set as 1. A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0229>.

Identification of a functional VDRE in Dtna-M promoter

Although there are representative examples of genes that are regulated by VDR in a VDRE-independent manner, the majority of VDR-upregulated genes are found to contain a VDRE sequence (Carlberg & Campbell 2013). We, therefore, aimed to identify the functional VDRE responsible for Dtna-M activation. The consensus sequences recognized

by VDR/RXR heterodimer or VDR homodimer are very similar; however, the presence of RXR increases the binding affinity of the target to VDRE (Nishikawa *et al.* 1994). We used PCR in order to produce truncated versions of Dtna-M construct, and the newly generated fragments containing progressively decreased numbers of putative VDREs recognized by VDR:RXR heterodimers were sub-cloned into the pGL4 vector (Fig. 6A). Dual luciferase assay conducted for Dtna-M full length and the three Dtna truncated constructs (Dtna-1746, Dtna-1530, and Dtna-1418) indicated loss of induction for the shortest Dtna-1418 construct only (Fig. 6A). To further ascertain that the absence of induction in Dtna-1418 upon VD3 administration is due to the missing (-1474/-1461) VDRE and not due to an alteration of the character of the Dtna-M promoter attributed to the truncation, we have generated mutants that tampered with the individual putative VDREs located near the -1474/1461 region (Fig. 6B). Dual luciferase assay confirmed that loss of induction occurred only for the Mut (-1473/-1471) and not for the upstream Mut (-1538/-1536). Furthermore, mutation of the VDR homodimer binding region (-968/-952) located downstream of the (-1474/-1461) VDRE did not affect the induction of Dtna-M by VD3 (data not shown). Our results indicate that deletion of (-1474/-1461) VDRE region (clearly depicted on Fig. 6C) only alters the responsiveness of Dtna-M to VD3.

To double confirm the functionality of our newly identified VDRE in Dtna-VD3 mediated response, we have performed ChIP assay using primers to amplify a 100 bp fragment of Dtna-M promoter encompassing the (-1474/-1461) VDRE region and three different VD3 concentrations (Fig. 6D). Under all conditions of VD3 supplementation, we have proved that VDR is recruited to the VDRE binding site of our Dtna-M promoter. Primers encompassing the other two *in silico* identified putative VDRE binding sites (-1546/1544 and -1473/1471) were also used but did not produce any band when using template from VD3 supplemented samples (data not shown). Thus, we can safely conclude that Dtna-M promoter response to VD3 in our system is VDR mediated.

Discussion

Since the identification of a functional VDR in muscle cells, the research field aiming to discover muscle-specific genes that are modulated by VD3/VDR axis has rapidly expanded. A biphasic mode of VD3-mediated regulation of *in vitro* myogenesis has been proposed in a sense that, while VD3 administration throughout the course of myogenic

Figure 6

Identification of a functional VDRE in Dtna-M promoter. (A) Schematic representation of Dtna-M truncated luciferase constructs illustrating their respective putative VDRE as identified via *in silico* analysis. Dual luciferase assay in early myotubes indicate loss of Dtna-M VD3-dependent induction for the shortest Dtna-1418 construct only. (B) Schematic representation of individual VDRE region deletion constructs through mutagenesis of three key amino-acids (position noted using red square) and dual luciferase activity assay performed in C2C12 early myotubes indicate abolishment of Dtna-M-VD3 induction in Mut (-1473/-1471) only, confirming the truncation data. The Dtna-M and/or truncated/mutated constructs were transfected in C2C12 cells and assayed as described in Fig. 5. Data are expressed as mean $\pm$ s.e.m. $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$. $n = 3$ separate experiments. Comparisons between groups were done using 2-way ANOVA with Sidak's post-hoc test. (C) VDREs typically consist of two conserved hexameric half-sites separated by a three nucleotide spacer as shown on the left (retrieved from JASPAR). Chromatograms indicating the functional VDRE sequence in Dtna-M as well as the mutated VDRE sequence in Mut (-1473/-1471) that disrupts hexameric pattern are displayed on the right (native and mutated triplet is denoted by a red rectangle). (D) CHIP-PCR amplification demonstrates binding of VDR to the VDRE region that was deleted in mutant Mut (-1473/-1471) in C2C12 cells upon administration of 10, 25, and 50 nM VD3 for 24 h. PCRs were performed including input positive control (2%) using primers binding to the Dtna promoter deleted region and Gapdh promoter region. NT: no VD3 treated; IgG negative controls. $n = 2$ separate experiments.

differentiation may increase myoblast proliferation and delay myotube formation, myotubes treated with high doses of VD3 have increased size and diameter (Girgis *et al.* 2014a, van der Meijden *et al.* 2016). Non-genomic

effects of VD3 action are also described (Hii & Ferrante 2016), that could involve the presence of an existing, yet unidentified membrane VDR receptor (Marcinkowska 2001). Such representative non-genomic VD3 actions are the activation of p38 MAPK and phospholipase C pathways by VD3 that ultimately regulate myoblast proliferation and myogenic differentiation (Wu *et al.* 2000, Jones *et al.* 2001). The mode of cross-talk between these existing VD3-mediated pathways relevant to myogenesis remains hard to untangle.

In human, similarly to mouse, *in situ* VDR expression levels in skeletal muscle was inversely correlated with age, but no alteration in serum VD3 levels was observed in elderly individuals (Bischoff-Ferrari *et al.* 2004). Low VD3 serum levels have been associated with lower limb strength (Hassan-Smith *et al.* 2017), whereas low 25(OH)D level, routinely assayed in serum in order to assess vitamin D status, was linked to proximal muscle weakness or muscle discomfort (Plotnikoff & Quigley 2003, Rejnmark 2011). Clinical data suggest a positive correlation between VD3 and muscle mass improvement along with a reduced risk of falls in the elderly population (Snijder *et al.* 2006). Vitamin D depletion is associated with fast fiber muscle atrophy and musculoskeletal abnormalities (Boland 1986, Sato *et al.* 2005), but clinical studies failed to show regulation of muscle-specific genes via VD3 so far. It is speculated that heterogeneity of VD3 treatment regime prescribed worldwide may be responsible for conflicting outcomes among clinical trials. Establishing a suitable VD3 dosage for individual patients tailored to their specific condition remains a very challenging task.

Herein, we have identified *Dtna*, a homologue of dystrophin and a key component of DAPC, as a gene induced explicitly by VD3 and confirmed that this induction is VDR mediated and specific. Administration of low doses of VD3 during individual phases of myogenic differentiation in the presence of VDR upregulated *Dtna* gene and protein expression in healthy myotubes. Experiments in H2K-mdx52 cells indicated that DTNA upregulation by VD3 can occur in the absence of dystrophin, possibly aiding the stabilization of the complex. It would be interesting to examine whether proteins closely interacting with DTNA, such as syntrophins and aquaporin, are rescued by VD3-mediated DTNA upregulation.

It is worth mentioning that the functionality of the VDRE element identified in our Dtna-M promoter was dependent on the potential of C2C12 cells to differentiate to myotubes. For instance, when we performed dual luciferase assay using individual VDRE-designed deletion constructs in C2C12 myoblasts, all of them with the

exception of Mut (-1474/-1461) were induced by VD3 presence (Fig. 7A). However, when we transfected the same mutation or truncation constructs in Neuro2a cells, all VD3-treated constructs exhibited high activity (Fig. 7B). We therefore cannot exclude that, to some extent, interaction with myogenic or muscle-specific factors may partially modulate the induction of Dtna-M promoter or that VDR expression may be selectively regulated in cells or tissues of different origin, creating a different environment for gene targets to bind (Pike 2014).

Another finding we wish to comment on is the demonstration that, at least *in vitro*, the fast fiber marker upregulation by VD3 in myotubes was dependent on the presence of VDR, whereas the slow fiber marker

upregulation occurred irrespective of the VDR presence on the system, hinting that intermediate pathways, such as the MAPK pathway, may be responsible for such increase. While an increase in fast (type II) fibers may prove to be beneficial for the elderly population, because they are associated with higher ability of individuals to quickly adjust to posture perturbations (Paillard 2017), an increase in slow (type I) fibers is instead considered to be therapeutic for dystrophic patients (Boyer *et al.* 2019). Therefore, a postulated fast-to-slow fiber type switching by VD3 in adult skeletal muscle is worth exploring *in vivo*.

As a next step, we are keen to investigate the effect of low doses of VD3 administration and *Dtna* potential modulation in the *mdx* and *mdx52* mice models, where altered pattern of degeneration/regeneration leads to exhaustion of satellite cell pool and progressive muscle damage. The *mdx* mouse model harbors a nonsense mutation in exon 23, that results in an early termination codon and a truncated, non-functional dystrophin. The *mdx* mouse exhibits pathophysiological hallmarks present in DMD such as myofiber necrosis and rapid regeneration/degeneration cycles; however, phenotypic characteristics such as muscle weakness and fibrosis are mild and lifespan is not reduced (Sicinski *et al.* 1989). The *mdx52* mouse was generated via targeted deletion of exon 52 and lacks the Dp427 skeletal isoform as well as the shorter Dp260 and Dp140 isoforms that are present in the *mdx* mouse (Araki *et al.* 1997). Because the deleted 52 exon corresponds to the hotspot between exons 45–55 for human DMD patients, this so-called humanized model has been mainly used to evaluate exon skipping efficacy in dystrophinopathies (Yucel *et al.* 2018). In general, mice that lack *Dtna* exhibit a mild skeletal myopathy, with phenotype resembling a less severe inflammatory cell infiltration response than that observed in *mdx* model, attributed to the destabilization of DAPC and impaired nNOS signalling, albeit without evident functional deficits (Grady *et al.* 1999, Bunnell *et al.* 2008). DTNA levels are significantly reduced at the sarcolemma of *mdx* mice (Blake 2002), *mdx52* mice (our group's unpublished proteomic analysis data), or mice lacking the sarcoglycan complex (Strakova *et al.* 2014). DTNA impairment was also confirmed in DMD patients and limb-girdle muscular dystrophy patients with mutations in sarcoglycan components, indicating that DTNA may play an active role in the pathogenesis of muscular dystrophies (Metzinger *et al.* 1997). In DMD patients, the reduced levels of Dtna1 and syntrophin alpha 1 in muscle biopsies were attributed to the inability of these two DAPC components to properly localize to the sarcolemma, due to the disturbed dystrophin anchorage

Figure 7

Dtna-M response to VDR through the VDRE is strongly dependent on the ability of cells to differentiate as well as on the presence of myogenic factors. (A) Dual luciferase activity assay performed in C2C12 myoblasts for individual VDRE mutation constructs indicate Dtna-M –VD3 induction for all except for the Mut (-1473/-1471), confirming previous data. (B) When dual luciferase activity were performed for Dtna-M truncation and VDRE mutation constructs in Neuro2a cells that do not have the capacity to differentiate, no abolishment of Dtna-M induction was observed. Dual luciferase activity was measured in cell monolayers incubated in GM in presence of vehicle or VD3 (10 nM) for 24 h (Neuro2a cells) or 48 h (C2C12 cells), and firefly luciferase activity was normalized to renilla luciferase activity. Data are expressed as mean $\pm$ s.e.m. * $P < 0.05$ and **** $P \leq 0.0001$. $n = 3$ separate experiments. Comparisons between groups were done using 2-way ANOVA with Sidak's post-hoc test. A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0229>.

(Compton *et al.* 2005). Aberrant *Dtna* splicing may interfere with syntrophin binding in myotonic muscular dystrophy, indicating potential significance of *Dtna* in muscle, although no *Dtna* mutation has been identified in patients so far (Nakamori & Takahashi 2011). Raising DTNA levels may constitute a complementary treatment in order to reinforce dystrophin restoration in gene therapy interventions for dystrophinopathies.

In conclusion, we have shown that VD3-VDR pathway upregulates *Dtna* gene DTNA protein expression and found a functional VDRE in the muscle-specific promoter region of murine *Dtna* gene. These results suggest that VD3-VDR pathway has a beneficial effect on dystrophic muscle which should be examined in the future studies.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-19-0229>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Conceptualization was performed by M K T, S F, and T M; methodology by M K T, S S, M I, and S F; investigation by M K T; writing and original draft by M K T; review and editing by M K T, S F, S T, T M, and Y A; funding acquisition by M K T, T M, and Y A; resources were obtained by T M and Y A; and supervision by T M, S F and Y A.

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